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(21) International Application Number: PCT/US90/03138 (22) International Filing Date: 5 June 1990 (05.06.90) (30) Priority data: 361,045 5 June 1989 (05.06.89) US (60) Parent Application or Grant (63) Related by Continuation US 361,045 (CIP) Filed on 5 June 1989 (05.06.89) (71) Applicant (for all designated States except US): GILEAD SCIENCES, INC. [US/US]; 344 Lakeside Drive, Foster City, CA 94404 (US).		(72) Inventor; and (75) Inventor/Applicant (for US only) : FROEHLER, Brian, C. [US/US]; 2310 Monserat Avenue, Belmont, CA 94002 (US). (74) Agents: REED, Dianne, E. et al.; Irell & Manella, 545 Middlefield Road, Suite 200, Menlo Park, CA 94025 (US). (81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent), US. Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: EXONUCLEASE-RESISTANT OLIGONUCLEOTIDES AND METHODS FOR PREPARING THE SAME (57) Abstract <p>A method is provided for making 3' and/or 5' end-capped oligonucleotides so as to render the oligonucleotide resistant to degradation by exonucleases. The exonuclease degradation resistance is provided by incorporating two or more phosphoramidate and phosphoromonothioate and/or phosphorodithioate linkages at the 5' and/or 3' ends of the oligonucleotide, wherein the number of phosphoramidate linkages is less than a number which would interfere with hybridization to a complementary oligonucleotide strand and/or which would interfere with RNaseH activity when the oligonucleotide is hybridized to RNA.</p>		

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5 EXONUCLEASE-RESISTANT
 OLIGONUCLEOTIDES AND
 METHODS FOR PREPARING THE SAME

Description

10

Technical Field

 The present invention is directed to
 oligonucleotides containing a 3'- and/or 5'-capped
 terminal and which are thereby rendered resistant to
15 degradation by exonucleases. The exonuclease-resistant
 oligonucleotides have two or more phosphoramidate
 internucleotide linkages at one or both termini which
 render the oligonucleotides resistant to degradation.

20 Background

 DNA molecules contain internucleotide
 phosphodiester linkages which are degraded by
 exonucleases present in cells, culture media and human
 serum. For example, degradation by exonucleases in
25 tissue culture media of DNA may be observed within about
 30 minutes to about six hours. Synthetic oligodeoxy-
 nucleotides with phosphodiester linkages are routinely
 used in genetic engineering, for example, to locate
 specific RNA or DNA fragments from a library. The long-
30 term stability of an oligonucleotide for this utility is
 not a major concern, since the oligonucleotide is usually
 not exposed to the relatively stringent environment of
 the culture medium, therefore exonuclease degradation is
 not a substantial problem.

35 However, it is in fact frequently desirable to
 produce oligodeoxynucleotides which are stable (i.e., for
 more than several hours or days) for long-term uses. For

example, a oligodeoxynucleotide with phosphodiester linkages can be used to block protein synthesis by hydrogen bonding to complementary messenger RNA thereby providing a tool for use in an antisense fashion.

- 5 Exonuclease-stable oligodeoxynucleotides could also be utilized to form triple-helix DNA which would interfere with the transcription process or with DNA replication, by competing with naturally occurring binding factors or by gene destruction. However, in order to utilize
10 synthetic oligonucleotides in this manner, they must be stable to exonucleases, the major activity of which in cells and serum appears to be 3' to 5', i.e., digestion of oligonucleotides begins starting at the 3' end.

The present invention is accordingly directed
15 to such exonuclease-stable oligonucleotides.

Related Art:

The following references relate to one or more aspects of the presently claimed invention:

- 20 Froehler, Tet. Lett. 27(46):5575-5578 (1986), describes polymer-bound deoxynucleoside H-phosphonate diesters as precursors to phosphoramidate, thiophosphate and phosphate triester analogs of DNA.

- 25 Froehler et al., Nuc. Acids Res. 16(11):4831-4839 (1988), describe the synthesis of a 15-mer containing 12 phosphoroamidate linkages derived from primary and secondary amines. The chemistry of the process is summarized in the figure shown on page 4833 of the reference.

- 30 Froehler et al., Nuc. Acids Res. 14(13):5399-5407 (1986), describe the synthesis of deoxyoligo-nucleotides via deoxynucleoside H-phosphonate intermediates. The chemistry of this process is essentially shown in scheme 2 on page 5401 of the
35 reference.

Froehler, European Patent Publication No. 219342-A2, published 2 April 1987, is similar to the teachings of the latter two references in that the synthesis of DNA via deoxynucleoside H-phosphonate intermediates is shown.

Letsinger et al., Nuc. Acids Res. 14(8):3487-3499 (1986), describe complexes of polyuridylic acid (poly U) and polythymidylic acid (poly dT) with oligonucleotides possessing different pendant groups that are linked to the oligonucleotide chain at the internucleotide phosphodiester linkages.

Stein et al., Nuc. Acids Res. 16(8):3209-3221, (1988) present a study of oligodeoxynucleotides modified so as to contain phosphorothioate linkages. The authors, in addition to evaluating a number of other physico-chemical properties of such oligonucleotides, study the susceptibilities of the compounds to a number of endonucleases and exonucleases. The authors found a significant decrease in the T_m of fully substituted phosphorothioate oligodeoxynucleotides compared to diester controls (Figure 3), i.e., a 15-20°C decrease in T_m and a 30-40 Kcal/mole decrease in ΔH for fully substituted molecules (p. 3215).

Brill et al., Tet. Lett. 29(43):5517-5520 (1988) describe the preparation of dinucleoside phosphorodithioates by sulfur oxidation of thiophosphate triesters.

Agrawal, Tet. Lett. 28(31):3539-3542 (1987) describe the automated synthesis of oligodeoxynucleosides containing methylphosphonate linkages, using nucleoside methylphosphonamidites as starting materials. The authors conclude that two adjacent methylphosphonate linkages at the 3' end provides protection against degradation by snake venom phosphodiesterase and spleen phosphodiesterase (and, like Stein et al., the authors do

not evaluate nuclease stability of the oligonucleotides in serum, tissue culture medium or cells).

PCT publication W089/05358, inventors Walder et al., describe oligodeoxynucleotides modified at the 3' terminus so as to render the oligonucleotide chain resistant to degradation within cells and body fluids. Disclosed modifications at the 3'-terminal phosphodiester linkage include replacement of that linkage with an alkyl or aryl phosphotriester, hydrogen phosphonate, an alkyl or aryl phosphonate, an alkyl or aryl phosphoramidate, a phosphorothioate, or a phosphoroselenate, although the preferred modification is stated to be the incorporation of a 3'-terminal phosphotriester linkage.

15 Disclosure of the Invention

Accordingly, it is a primary object of the invention to address the above-mentioned need in the art and to provide exonuclease-resistant oligonucleotides.

It is another object of the invention to provide exonuclease-resistant oligonucleotides modified at the 3'-terminus so that the initially present phosphodiester linkages are replaced with a specified number of phosphoramidate linkages.

It is still another object of the invention to provide such exonuclease-resistant oligonucleotides which additionally contain phosphoromonothioate and/or phosphorodithioate linkages.

It is yet another object of the invention to provide exonuclease-resistant oligonucleotides which are capable of hybridizing to a complementary oligonucleotide strand.

It a further object of the invention to provide methods of making such exonuclease-resistant oligonucleotides.

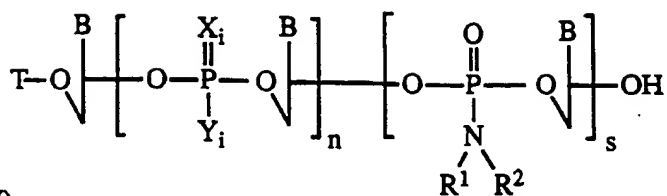
It is still a further object of the invention to provide a method for end-capping oligonucleotides with moieties which can perform multiple functions, such as aiding in transport, serving as chromophoric tags, or
5 enabling cross-linking.

Additional objects, advantages and novel features of the invention will be set forth in part in the description which follows, and in part will become apparent to those skilled in the art upon examination of
10 the following, or may be learned by practice of the invention.

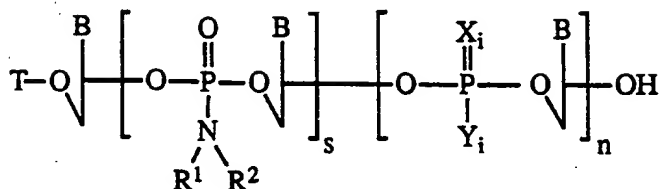
In a first aspect, the present invention provides oligonucleotides having two or more phosphoramidate linkages at the 3' terminus and/or 5'
15 terminus, which oligonucleotides are resistant to exonuclease degradation. The number of phosphoramidate linkages is at least 1 and less than a number which would interfere with hybridization to a complementary oligonucleotide strand, and/or less than a number which would
20 interfere with RNase activity when said oligonucleotide is hybridized to RNA. Preferably, at least 2, and more preferably on the order of about 2 to 10, phosphoramidate linkages are incorporated at either or both the 3' terminus and the 5' terminus. The phosphoramidate
25 linkages may be substituted with any one of a number of different types of moieties as will be described in detail hereinbelow.

In another aspect, exonuclease-resistant are provided which have the following formulas I, II or III,
30 i.e., containing phosphoramidate linkages as just described as well as phosphoromonothioate and/or phosphorodithioate linkages:

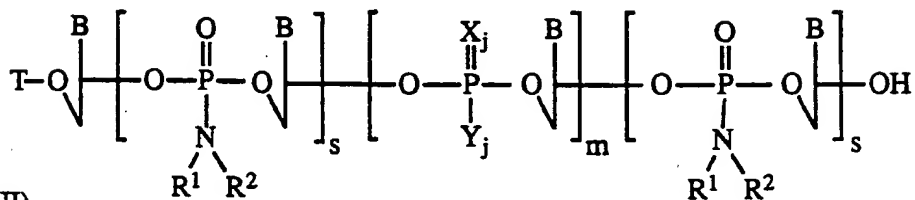
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(I)



(II)



(III)

wherein each n , m , i , j and s is independently an integer and each s is in the range of about 2 to 10; each n and m is independently from 1 to about 50; $s + n$ in formulas I and II is less than 100; and $s + s + m$ in formula III is less than about 100; each i varies from 1 to n ; each j varies from 1 to m ; T is hydrogen or a hydroxyl-protecting group; R^1 and R^2 are moities independently selected from the group consisting of hydrogen, hydrocarbyl substituents of 20 carbon atoms or less, and

oxyhydrocarbyl of 20 carbon atoms or less and 1-3 oxy groups, wherein said hydrocarbyl and oxyhydrocarbyl substituents are linear or branched alkyl of 1 to 20 carbon atoms, linear or branched alkenyl of 2 to 20 carbon atoms, cycloalkyl or cycloalkenyl of 3 to 20 carbon atoms, linear or branched alkoxy of 1 to 20 carbon atoms, or aryl of 6 to 18 carbon atoms, with the proviso that R^1 and R^2 are not both hydrogen;

each B is independently a protected or unprotected heterocyclic base;

each X_i and X_j is independently O or S; and

each Y_i and Y_j is independently R, -SR or -OR, where R is as defined for R^1 and R^2 .

The present invention also provides methods for preparing such end-capped oligonucleotides.

Modes for Carrying Out the Invention

As used herein the terms "polynucleotide" and "oligonucleotide" shall be generic to polydeoxyribonucleotides (containing 2'-deoxy-D-ribose or modified forms thereof), to polyribonucleotides (containing D-ribose or modified forms thereof), and to any other type of polynucleotide which is an N-glycoside of a purine or pyrimidine bases, or modified purine or pyrimidine bases. The term "nucleoside" will similarly be generic to ribonucleosides, deoxyribonucleosides, or to any other nucleoside which is an N-glycoside of a purine or pyrimidine base, or modified purine or pyrimidine base. There is no intended distinction in length between the term "polynucleotide" and "oligonucleotide" and these terms will be used interchangeably.

It will be appreciated that as used herein the terms "nucleoside" and "nucleotides" will include those moieties which contain not only the known purine and pyrimidine bases, i.e., adenine, thymine, cytosine,

guanine and uracil, but also other heterocyclic bases which contain protecting groups or have been otherwise modified or derivatized.

By "modified nucleosides" or "modified nucleotides" as used herein are intended to include those compounds containing one or more protecting groups such as acyl, isobutyryl, benzoyl, or the like, as well as any of the wide range of modified and derivatized bases as known in the art. Examples of such modified or derivatized bases include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-ethylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 3-(3-amino-3-N-2-carboxypropyl) uracil, and 2,6-diaminopurine.

Modified nucleosides or nucleotides can also include modifications on the sugar moiety, for example, wherein one or more of the hydroxyl groups are replaced with halogen or aliphatic groups, or functionalized as ethers, amines, etc.

The polynucleotides according to the present invention may be of any length, but lengths of about

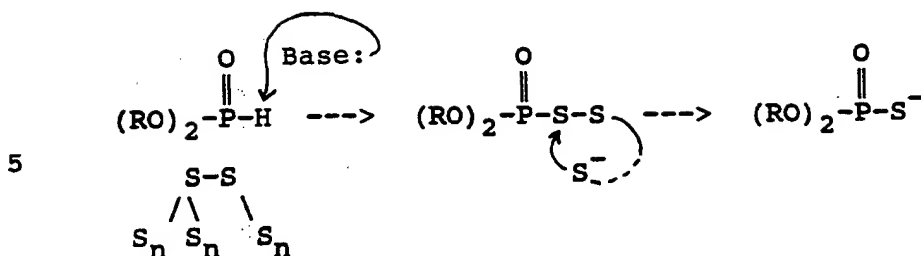
three to about fifty nucleotides are particularly useful for most genetic engineering applications. According to the present invention, the 3' end and/or the 5' end of the polynucleotide will contain at least two

5 phosphoramidate internucleotide linkages. The remaining internucleotide linkages may be phosphodiester linkages, phosphorothioate linkages or phosphorodithioate linkages, or any other internucleotide linkage, other than a phosphoramidate, or combinations of these other linkages.
10 Methods for preparing such non-phosphoramidate linkages are known in the art, e.g., as taught by Froehler et al., Nuc. Acids Res. 14:5399-5467 (1986), and Froehler, B., Tet. Lett. 27:5575-5578 (1986), cited above and incorporated by reference herein.

15 Internucleotide phosphodiester linkages are prepared from hydrogen phosphonate linkages preferably by oxidation with, e.g., aqueous iodine. A typical procedure involves treatment of the hydrogen phosphonate in 0.1 M iodine in Pyr/NMI/H₂O/THF (5:1:5:90) for about
20 2-3 minutes, followed by treatment with 0.1 M iodine in Et₃/H₂O/THF (5:5:90) for another approximately 2-3 minutes.

Phosphoromonothioate linkages are formed from the initially present hydrogen phosphonate linkages by
25 treatment with sulfur. The reaction is carried out at approximately room temperature for on the order of 20 minutes in a solvent system which typically includes a sulfur solvent such as CS₂ along with a basic solvent such as pyridine. Other suitable solvent systems include
30 CS₂/lutidine and CS₂/triethylamine; CS₂ is preferred as the sulfur solvent because it acts to dissolve elemental sulfur. The following scheme illustrates the postulated reaction:

35



10 (See, e.g., Stein et al., cited above.)

To form hydrogen phosphorodithioate linkages, sulfurization of the hydrogen phosphoromonothioate linkages is effected using conditions identical to those just described for the preparation of the phosphoromono-

15 thioate moiety. (Note: the term "phosphorothioate" as used herein is intended to encompass both "phosphoromonothioate" and "phosphorodithioate" linkages.)

Structure of the End-Capped Oligonucleotides:

20 The oligonucleotides of the invention, as noted above, are resistant to degradation under both physiological and tissue culture conditions, and in particular are resistant to degradation by exonucleases.

In order that the oligonucleotide be resistant

25 to such enzymatic degradation, it is modified so that phosphodiester linkages initially present at the 3' terminus are replaced with a selected number of phosphoramidate linkages, that number being at least one and less than a number which would cause interference with

30 hybridization to a complementary oligonucleotide strand, and/or less than a number which would interfere with RNaseH activity when said the oligonucleotide is hybridized to RNA. Such a modification may additionally or alternatively be made at the 5' terminus.

35 It is preferred that the number of phosphoramidate linkages be selected such that the melting temperature of any duplex formed with complement is

lowered by less than about 10°C relative to that obtained with an oligonucleotide containing only the initial phosphodiester linkages. Preferably, the number of phosphoramidate linkages is such that the melting
 5 temperature of a duplex formed is lowered by less than about 5°C. The number of phosphoramidate linkages present is typically and preferably between about 2 and 10, more preferably between about 2 and 8, and most preferably between about 2 and 6.

10 The phosphoramidate linkage has the formula



20 wherein the R¹ and R² moieties are substituents which must be selected so as not to interfere with hybridization with complement. In most cases, the groups R¹ and R² are independently selected from the group consisting of hydrogen, hydrocarbyl substituents of
 25 20 carbon atoms or less, and oxyhydrocarbyl substituents of 20 carbon atoms or less containing 1-3 oxy groups, with the proviso that R¹ and R² are not both hydrogen, i.e., the phosphoramidate linkages herein are always N-substituted. In this case, it is preferred that one of
 30 the two substituents be hydrogen. Suitable hydrocarbyl and oxyhydrocarbyl substituents include, for example, linear or branched alkyl of 1-20 carbon atoms, linear or branched alkenyl of 2-20 carbon atoms, cycloalkyl or cycloalkenyl of 3-20 carbon atoms, linear or branched
 35 alkoxy of 1-20 carbon atoms, or aryl of 6-18 carbon atoms. The hydrocarbyl substituent may be, for example, an alkoxy substituent having the formula CH₃O-(CH₂)_x- or a straight chain alkyl group having the formula

$\text{CH}_3(\text{CH}_2)_y-$ where x is an integer in the range of 1-20, inclusive, preferably in the range of 1-10, inclusive, and y is an integer in the range of 0-15, inclusive.

5 Examples of preferred oligonucleotide linkages within the aforementioned groups are wherein one of R^1 and R^2 is H and the other is either 2-methoxyethyl, dodecyl, or *n*-propyl. (The 2-methoxyethyl and dodecyl linkages are sometimes referred to herein as "MEA" and "C12", respectively.)

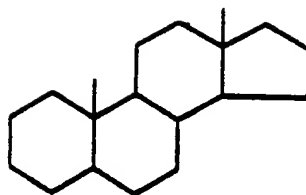
10 The R^1 and R^2 groups may also be, in addition to the foregoing, macromolecular species such as sugars, polypeptides, chromophoric groups, lipophilic groups, polymers, steroid hormones, or the like.

"Lipophilic" groups refer to moieties which are
15 chemically compatible with the outer cell surface, i.e., so as to enable the oligonucleotide to attach to, merge with and cross the cell membrane. Examples of such lipophilic groups are fatty acids and fatty alcohols (in addition to the long chain hydrocarbyl groups described
20 above).

Examples of preferred polypeptides that can be used for R^1 and/or R^2 include transferrin and epidermal growth factor (EGF), while suitable non-polypeptide
25 polymers include ionic, nonionic and zwitterionic polymers. Examples of a particularly preferred polymer is polyethylene glycol.

Steroid substituents include any of the general family of lipid compounds which comprise sterols, bioacids, cardiac glycosides, seponans, and sex hormones,
30 which include the following basic structure:

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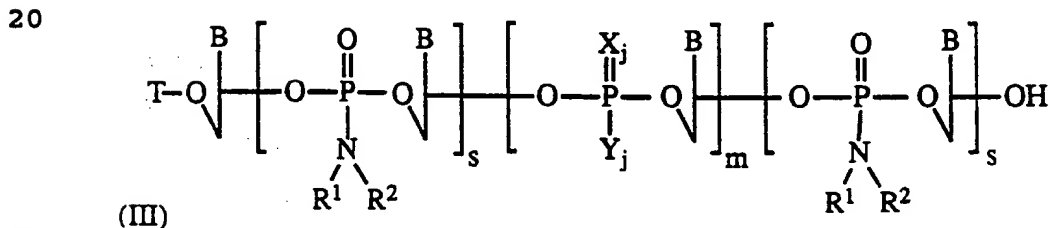
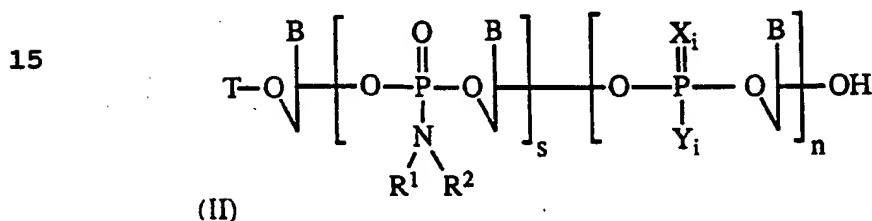
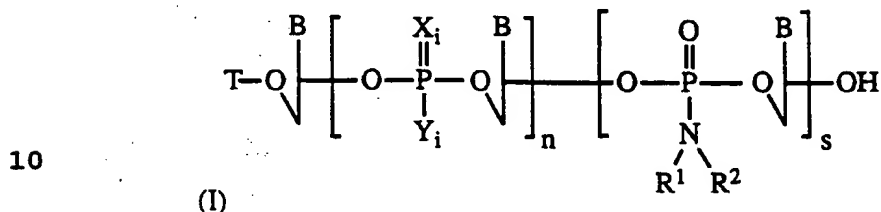


10 Examples of steroids include natural corticosteroid hormones (produced by the adrenal glands), sex hormones (progesterone, androgens, and estrogens).

These various R^1 and R^2 groups can confer any of a variety of desired properties to the oligo-
15 nucleotide. For example, if R^1 or R^2 is a polymer such as polyethylene glycol, a polypeptide or a lipophilic group such as a long-chain hydrocarbyl moiety, such a group may facilitate transport or permeation of the oligonucleotide through cell membranes, thus increasing
20 the cellular uptake of the oligonucleotide. The R^1 or R^2 group may also be a group which affects target DNA or RNA to which the oligonucleotide will bind, such as providing covalent linkages to the target strand to facilitate cleavage or intercalation of the oligonucleotide to the
25 target strand. The R^1 and R^2 groups may additionally serve a cutting function (e.g., a site for cutting the complementary strand), or a receptor function (e.g., a receptor ligand).

It will be appreciated by those skilled in the
30 art that the oligonucleotides of the present invention can include other phosphoramidate N-substituents not explicitly disclosed herein so long as those substituents confer exonuclease resistance and do not interfere with hybridization to a complementary oligonucleotide strand.

The invention also encompasses oligonucleotide compositions containing oligonucleotides of the following formula I, II or III, i.e., wherein phosphoromonothioate and/or phosphorodithioate linkages are incorporated in addition to the phosphoramidate linkages:

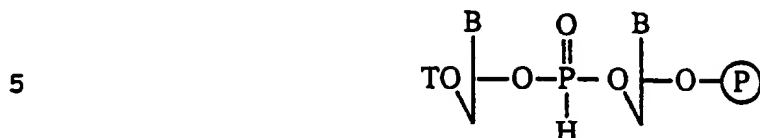


in which B, T, R¹, R², X_i, X_j, Y_i, Y_j, n, m, i, j and s are as defined above. In these structures, it is preferred that "s," which defines the number of phosphoramidate linkages, be in the range of 2-8, more preferably in the range of 2-6. It is also preferred that m and n be within the aforementioned ranges.

Synthetic Methods:

According to one embodiment of the present invention, the 3'-capped oligonucleotides may be prepared

by first preparing a polymer-bound polynucleoside with the formula IV



(IV)

10 wherein P is a solid state polymeric support, or other type of solid support, and B the base portion of a nucleoside, i.e., a purine or pyrimidine base, or any modified purine or pyrimidine base. As is conventional in oligonucleotide syntheses, the functional groups on
15 the base, i.e., the amine groups, will be appropriately protected during the course of the synthesis and removed after the completed polynucleotide is removed from the polymer support. As is the convention, in the formula shown above in IV, the linkage to the polymer support is
20 through the 3' hydroxy group, the free hydroxy group is the 5' group of the nucleoside. The group T is a conventional hydroxy-protecting group used in oligonucleotide synthesis, preferably the DMT group (dimethoxytrityl) or MMT group (monomethoxytrityl).
25 The polymer-bound polynucleoside hydrogen phosphonate (IV) is preferably prepared by treating the DBU (1.8-diazabicyclo[5.4.0]undec-7-ene ammonium salt) of a 5'-protected (preferably, 5 DMT) nucleoside hydrogen phosphonate with a polymer-bound nucleoside, linked to
30 support through its 3'-hydroxyl group in the presence of an activating agent, as is known in the art. Methods for preparing such polymer-bound polynucleoside hydrogen phosphonates are disclosed, for example, by Froehler, B., et al., Nuc. Acids Res. 16:4831-4839 (1988); Froehler, B., et al., Nuc. Acids Res. 14:5399-5467 (1986); and
35

Froehler, B., et al., Nucleosides and Nucleotides 6:287-291 (1987). Then, one or more nucleoside hydrogen phosphonates may be added (to make the two or more internucleotide linkages at the 3' end of the polynucleotide) by sequentially deprotecting the 5'-hydroxyl group of the polymer-bound polynucleotide, and condensing with the next nucleoside hydrogen phosphonate. The oligonucleotide chain elongation will proceed in conformance with a predetermined sequence in a series of condensations, each one of which results in the addition of another nucleoside to the oligomer. The condensation is typically accomplished with dehydrating agents, which are suitably phosphorylating agents or acylating agents such as isobutylchloroformate, diphenylchlorophosphate, organic acid anhydrides (such as acetic anhydride, isobutyric anhydride or trimethyl acetic anhydride) and organic acid halides such as pivaloyl chloride, pivaloyl bromide, 1-adamantyl-carboxylic chloride or benzoyl chloride. The preferred condensing agent is pivaloyl chloride in pyridine acetonitrile. Prior to the addition of each successive nucleoside hydrogen phosphonate, the 5'-protecting group or the carrier bound nucleotide is removed. Typically, for removal of the DMT group, this is done by treatment with 2.5% volume/volume dichloroacetic acid/ CH_2Cl_2 , although 1% weight/volume trichloroacetic acid/ CH_2Cl_2 or ZnBr_2 -saturated nitromethane are also useful. Other deprotection procedures suitable for other known protecting groups will be apparent to those of ordinary skill in the art.

The carrier is preferably washed with anhydrous pyridine/acetonitrile (1/1,v/v) and the condensation reaction is completed in as many cycles as are required to form the desired number of 3'-end internucleotide bonds which will be converted to phosphoramidates. After

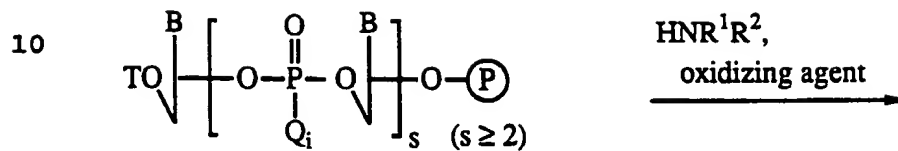
the required number of synthetic cycles, the carrier-bound polynucleotide hydrogen phosphonate is oxidized to convert the hydrogen phosphonate internucleotide linkages to phosphoramidate linkages, preferably by treatment with the desired amine NHR^1R^2 with R^1 and R^2 as defined earlier and CCl_4 as described in Froehler, et al., Nucleic Acids Research 16:4831-4839 (1988). Although carbon tetrachloride is preferred, other mild oxidizing agents may be utilized.

After the oxidation to form the phosphoramidate internucleotide linkages, the oligonucleotide is then completed by methods which form nonphosphoramidate linkages, such as phosphodiester linkages, phosphorothioate linkages or phosphorodithioate linkages, by methods known in the art referenced above and incorporated by reference herein. The preferred method for completing the oligonucleotide is to continue the sequence using 5'-protected nucleoside hydrogen-phosphonates. In the instance where the 5' end will not be capped, after the last 5'-protected nucleoside hydrogen phosphonate has been added, all of the hydrogen phosphonate linkages are oxidized to produce diester linkages, preferably by aqueous iodine oxidation or oxidation using other oxidizing agents, such as N-chlorosuccinimide, N-bromosuccinimide or salts or periodic acid. This will result in all of the internucleotide linkages, except for the 3'-end capped linkages which are phosphoramidate linkages, being phosphodiester linkages. Thereafter, the oligonucleotide may be separated from the carrier, using conventional methods, which in the preferred instance is incubation with concentrated ammonium hydroxide. Any protecting groups may be removed as described above using about 2% dichloroacetic acid/ CH_2Cl_2 , or about 80% acetic acid, or by other conventional methods, depending on the nature of

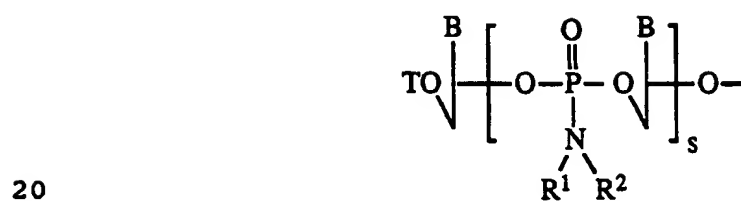
the protecting groups. The desired oligonucleotide is then purified by HPLC, polyacrylamide gel electrophoresis or using other conventional techniques.

5 The following schemes illustrate various synthetic processes within the scope of the invention:

5

Scheme 1

15



25

T: protecting group

Ⓟ: protecting group or solid state carrier

i: varies from 1 to 5

Q: hydrogen or $-\text{NR}^1\text{R}^2$ (with the proviso that
at least one Q_i is hydrogen)

30

B: a purine or pyrimidine base

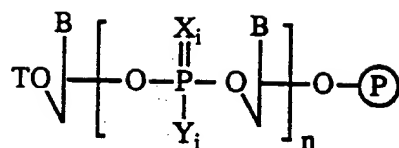
 R^1, R^2 : see text

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Scheme 2a

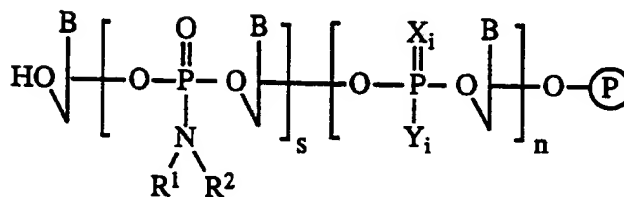
10

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- 1) 5' - blocked nucleoside H - phosphonate
and pivaloyl chloride as activator
- 2) remove 5' - blocking group
- 3) HNR^1R^2 /oxidizing agent
- 4) optional repetition of steps 1-3

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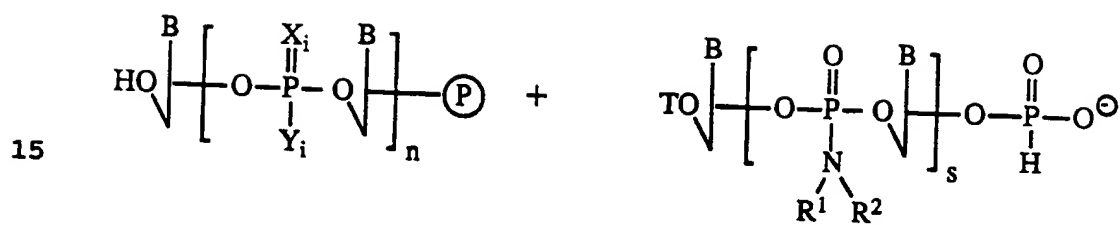
$\text{T}, \textcircled{\text{P}}, \text{X}_i, \text{Y}_i, \text{R}^1, \text{R}^2 =$ as defined in text and in Scheme 1

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Scheme 2b

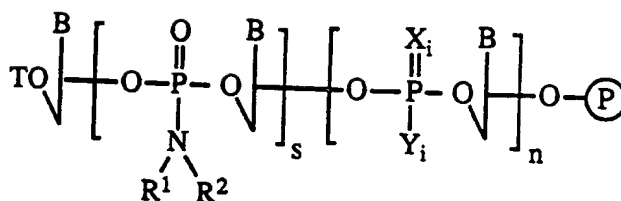
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5' - blocked nucleoside

20

- 1) activating agent
- 2) oxidation



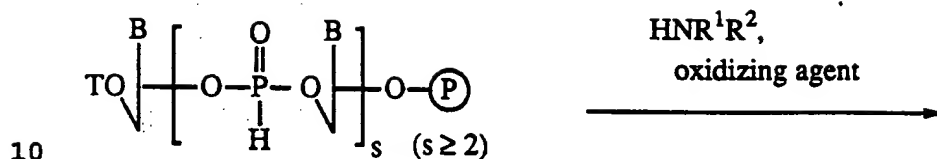
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T, X_i, Y_i, R¹, R², s, n, = as defined in text and in Schemes 1 and 2a

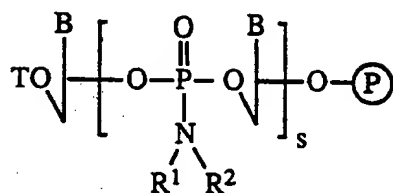
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Scheme 3

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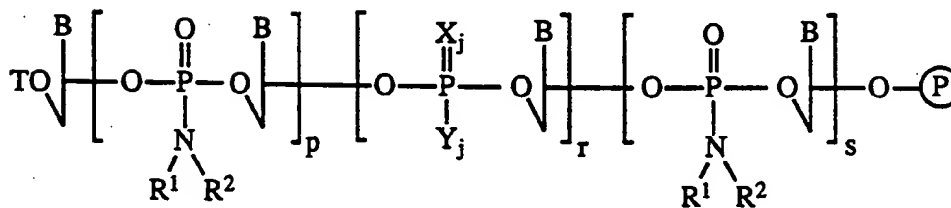
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- 1) remove T
- 2) condense 5' - blocked nucleoside H-phosphonate, H-phosphorothioate or H-phosphorodithioate with pivaloyl chloride as activator
- 3) remove 5' - blocking group
- 4) repeat steps 2) & 3)
- 5) oxidize to form phosphodiester, phosphorothioate and/or phosphorodithioate linkages
- 6) condense with additional 5' - blocked nucleoside phosphonates, optionally react with HNR^1R^2 in the presence of an oxidizing agent

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The foregoing discussion has revolved around the consecutive addition of mononucleoside hydrogen phosphonates, but it will be understood that one or more nucleotides can be added in a given cycle by using a polynucleotide, such as a di- or trinucleotide.

It will also be understood that while the above method has been described in connection with use of a solid state carrier if the object oligonucleotide is small, i.e., containing, for example, only five nucleotides (therefore having only four internucleotide linkages, two of which are phosphoramidate linkages) it is feasible to conduct the synthesis without the use of a solid state support. In such an instance a conventional 3'-hydroxy protecting group may be used which is different from the 5'-protecting group used in the synthesis, so that the 5'-protecting group may be selectively removed while the 3'-protecting group remains intact.

It will also be appreciated that the two or more phosphoramidate linkages need not each contain the same R^1 and R^2 groups. This may be accomplished by generating the first internucleotide hydrogen phosphonate linkage, and then oxidizing it with a first amine, generating the second hydrogen phosphonate internucleotide linkage, and then oxidizing it in the presence of a second (different) amine. This would result in a capped oligonucleotide having mixed phosphoramidate internucleotide linkages.

In another embodiment of the present invention, a 5'-capped oligonucleotide may be made. In such an instance, the above method may be modified by first forming a polymer-bound oligonucleotide having only hydrogen phosphonate internucleotide linkages which may then be oxidized to form phosphodiester (or phosphorothioate or phosphorodithioate linkages). Then

for the last two (or more) cycles, the 5'-end cap is formed when the last two or more nucleosides are added, followed by reaction with the amine NHR^1R^2 .

Alternatively, the 5' end may be added by adding a
5 polynucleotide, such as a tri- or tetranucleotide containing the desired phosphoramidate internucleotide linkages.

In still another embodiment, a combination of both of the above methods for making a 5' and a 3' end-capped oligonucleotide may be utilized. The first two
10 (or more) internucleotide linkages on the 3'-bound oligonucleotide may be oxidized to form the phosphoramidate linkages, then the non-terminal portion of the oligonucleotide may be made (having
15 phosphodiester, phosphorothioate or phosphorodithioate internucleotide linkages), with the final two (or more) linkages being phosphoramidates, formed as described above.

20 Methods of Use:

The uses of 5'- or 3'-phosphoramidate-capped oligonucleotides as made in accordance with the present invention may be as therapeutic agents against viral
diseases (such as HIV, hepatitis B, cytomegalovirus),
25 cancers (such as leukemias, lung cancer, breast cancer, colon cancer) or metabolic disorders, immune modulation agents, or the like, since the present end-capped oligonucleotides are stable within the environment of a cell as well as in extracellular fluids such as serum,
30 and can be used to selectively block protein synthesis, transcription, replication of RNA and/or DNA which is uniquely associated with the disease or disorder. The end-capped oligonucleotides of the invention may also be
used as therapeutics in animal health care, plant gene
35 regulation (such as plant growth promoters) or in human

diagnostics, such as to stabilize DNA probes to detect microorganisms, oncogenes, genetic defects, and the like, and as research reagents to study gene functions in animal cells, plant cells, microorganisms, and viruses.

5 There may also be dermatologic applications for treatment of diseases or for cosmetic purposes. There are many other potential uses which derive from the stability of the oligonucleotide to exonuclease degradation, thus prolonging oligonucleotide integrity within the

10 relatively stringent environment of the cells.

It is to be understood that while the invention has been described in conjunction with the preferred

15 specific embodiments thereof, that the foregoing description and the examples which follow are intended to illustrate and not limit the scope of the invention. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled

20 to which the invention pertains.

Example 1

Polymer-bound polynucleoside H-phosphonates were prepared as described by Froehler et al., supra, on control pore glass using the DBU salt of the protected nucleoside H-phosphonate. The diester linkages were generated by aqueous I_2 oxidation and the amidate linkages by amine/ CCl_4 oxidation. After two couplings the polynucleoside H-phosphonate was oxidized with a solution of 2-methoxyethylamine in Pyr/ CCl_4 (1:5:5) (20 min.) followed by twelve more couplings and oxidation with aq. I_2 (0.1 M in N-methyl morpholine/water/THF, 5:5:90) to generate a 15-mer containing two phosphoramidate linkages at the 3' end and twelve diester linkages. The oligomer was removed from the solid support, deprotected with conc. NH_4OH (45°C/18 hr.), and purified by HPLC (PRP) using an acetonitrile (CH_3CN) gradient in 50 mM aqueous TEAP. The DMT was removed from the product fraction (80% acetic acid/R.T./2 hrs.), evaporated, desalted and evaporated. Approximately 1 μg of purified product was 5' end-labeled with T4 polynucleotide kinase and γ - ^{32}P ATP for further characterization.

Example 2

Polymer-bound polynucleoside H-phosphonates were prepared as in the preceding example on control pore glass using the DBU salt of the protected nucleoside H-phosphonate. After twelve couplings the polynucleoside H-phosphonate was oxidized with aq. I_2 (0.1 M in N-methyl morpholine/water/THF, 5:5:90) followed by two more couplings and oxidation with a solution of 2-methoxyethylamine in Pyr/ CCl_4 (1:5:5) (20 min.) to generate a 15-mer containing twelve diester linkages at the 3' end and two phosphoramidate linkages at the 5' end. The oligomer was removed from the solid support, deprotected

with conc. NH_4OH (45°C/18 hr.) and purified by HPLC (PRP) using an acetonitrile (CH_3CN) gradient in 50 mM aqueous TEAP. The DMT was removed from the product fraction (80% acetic acid/R.T./2 hrs.), evaporated, desalted and
5 evaporated.

Example 3

Polymer-bound polynucleoside H-phosphonates were prepared as described as in the preceding examples
10 on control pore glass using the DBU salt of the protected nucleoside H-phosphonate. The diester linkages were generated by aqueous I_2 oxidation and the amidate linkages by amine/ CCl_4 . After two couplings the polynucleoside H-phosphonate was oxidized with a solution
15 of 2-methoxyethylamine in Pyr/ CCl_4 (1:5:5) (20 min.) followed by ten more couplings and oxidation with aq. I_2 (0.1 M in N-methyl morpholine/water/THF, 5:5:90) to generate a 13-mer containing two phosphoramidate linkages at the 3' end and ten diester linkages. This was
20 followed by two more couplings and oxidation with a solution of 2-methoxyethylamine in Pyr/ CCl_4 (1:5:5) (20 min.) to generate a 15-mer containing two phosphoramidate linkages at the 3' end, ten diester linkages, and two phosphoramidate linkages at the 5' end. The oligomer was
25 removed from the solid support and deprotected with conc. NH_4OH (45°C/18 hr.) and purified by HPLC (PRP) using an acetonitrile (CH_3CN) gradient in 50 mM aqueous TEAP. The DMT was removed from the product fraction (80% acetic acid/R.T./2 hrs.), evaporated, desalted and evaporated.

30

Example 4

The procedure of Example 1 was repeated using dodecylamine to generate a 15-mer containing two phosphoramidate linkages at the 3' end and twelve diester
35 linkages, wherein the phosphoramidate linkages are such

that one of R^1 and R^2 as defined earlier herein is hydrogen and the other is dodecyl.

Example 5

5 The procedure of Example 2 was repeated using dodecylamine in place of 2-methoxyethylamine, so as to yield a 15-mer containing twelve diester linkages at the 3' end and two phosphoramidate linkages at the 5' end, wherein the phosphoramidate linkages are substituted as
10 in the preceding example, i.e., one of R^1 and R^2 is hydrogen and the other is dodecyl.

Example 6

15 The procedure of Example 3 was repeated using dodecylamine in place of 2-methoxyethylamine, to give rise to a 15-mer containing two phosphoramidate linkages at the 3' end, ten diester linkages, and two phosphoramidate linkages at the 5' end, wherein the phosphoramidate is N-substituted as in the preceding two
20 examples.

Example 7

25 The procedure of Example 1 was repeated using propylamine to generate a 15-mer containing two phosphoramidate linkages at the 3' end and twelve diester linkages, wherein the phosphoramidate linkages are such that one of R^1 and R^2 as defined earlier herein is hydrogen and the other is n-propyl.

Example 8

30 The procedure of Example 2 was repeated using propylamine in place of 2-methoxyethylamine, so as to yield a 15-mer containing twelve diester linkages at the 3' end and two phosphoramidate linkages at the 5' end,
35 wherein the phosphoramidate linkages are substituted as

in the preceding example, i.e., one of R^1 and R^2 is hydrogen and the other is *n*-propyl.

Example 9

5 The procedure of Example 3 was repeated using propylamine in place of 2-methoxyethylamine, to give rise to a 15-mer containing two phosphoramidate linkages at the 3' end, ten diester linkages, and two phosphor-
10 amidate linkages at the 5' end, wherein the phosphor-
 amidate is N-substituted as in the preceding two examples.

Example 10

15 The following Example describes hybridization stability studies performed using end-capped oligonucleotides as described and claimed herein.

 Oligonucleotides containing end-caps were tested for their ability to form stable duplexes with complementary single-stranded DNA sequences; the various
20 oligonucleotides tested were outlined below in Table 1. Duplex stability was measured by determining the melting temperature T_m in solution over a range of temperatures. The experiment was conducted in a solution containing
25 150 mM NaCl, 5 mM Na_2HPO_4 and 3 μ M DNA at a pH of 7.1. The results obtained and set forth in Table 1 show that binding to complementary sequences is not materially affected by 3'-end-cap modification.

Table 1

[illegible]

Example 11

40 Several additional oligonucleotides also
end-capped at the 3' terminal two internucleotide
linkages were tested for their ability to form stable
duplexes with complementary single stranded DNA
sequences, as described in the preceding example.
45 Results are set forth in Table 2.

Table 2

5	Compound	T_m (°C)
10	<p>5' TCTCCCTCTCTTT-O-P(=O)(NHCH₂CH₂OCH₃)-O-T-O-P(=O)(NHCH₂CH₂OCH₃)-O-T-OH</p> <p>(methoxyethylamine)</p>	58.5
15		
20		
25	<p>5' TCTCCCTCTCTTT-O-P(=O)(O⁻)-O-T-O-P(=O)(O⁻)-O-T-OH</p>	59.5
30		
35	<p>5' TCTCCCTCTCTTT-OH</p> <p>(diester)</p>	56.5

Example 12

40 The following example was used to determine the efficacy of end-capped oligodeoxynucleotides virus inhibition and cellular toxicity using oligonucleotides capped at two terminal 3'-end internucleotide linkages with 2-methoxyethylamine and dodecylamine.

45 The acute infection assay used the MOLT-4 cell line which is susceptible to HIV infection. Measurement of HIV p24 was used to assay for inhibition of virus

replication 7 days after infection with virus at a multiplicity of infection of approximately 0.1. Approximately 1×10^6 cells were preincubated with oligonucleotide, washed, infected with virus stock and then incubated for 7 days in oligonucleotide. HIV p24 levels in the supernatant were measured by radioimmunoassay and compared with control infections lacking oligonucleotide. Results are expressed as the percent of control p24 found in cultures containing oligonucleotide. Sequences of antisense oligonucleotides were complementary to HIV targets listed in Table 3. Toxicity data was obtained by incubation of 3'-end-capped oligonucleotides with uninfected cells, followed by a comparison with cell numbers with control cultures incubated in the absence of oligonucleotide. Toxicity results are expressed as the percent reduction of cell numbers obtained by incubation in oligonucleotide for 7 days compared to controls. The effective inhibition of HIV replication using low levels (0.5 to 5 μM) of capped oligodeoxynucleotides supports the conclusion that significant nuclease degradation of the oligonucleotides of the invention does not occur either extracellularly or intracellularly.

Table 3

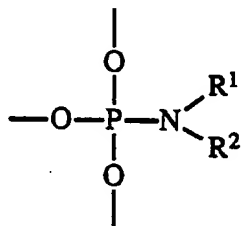
	<u>Target sequence</u>	<u>HIV Inhibition</u>	<u>Toxicity</u>
5	PBS		
	5'AGAGATTTTCCACAC3'		
	-methoxyethylamine		
	0.5 μ M	70%	0%
	5.0 μ M	90%	0%
10	50.0 μ M	--	4%
	-C12		
	0.5 μ M	0%	0%
	5.0 μ M	90%	2%
15	50 μ M	--*	5%
	NEF		
	5'TTGCCACCCATCTTA3'		
	-methoxyethylamine		
20	2.5 μ M	75%	0%
	5.0 μ M	80%	0%
	10.0 μ M	90%	0%
	50 μ M		0%
	100 μ M		0%
25	propylamine		
	2.5 μ M	65%	0%
	5.0 μ M	80%	0%
	50 μ M	--*	3%
30	100 μ M	--*	3%

* -- not done under HIV inhibition column.

Claims

1. An oligonucleotide resistant to degradation under physiological conditions, which oligonucleotide is modified so that the phosphodiester linkages at the 3' and/or 5' termini are replaced with phosphoramidate linkages, the number of said linkages being at least 1 and less than a number which would interfere with hybridization to a complementary oligonucleotide strand, and/or less than a number which would interfere with RNaseH activity when said oligonucleotide is hybridized to RNA.

2. The oligonucleotide of claim 1 wherein the phosphoramidate has the formula:



wherein R^1 and R^2 are substituents which do not interfere with hybridization with complement.

3. The oligonucleotide of claim 2 wherein R^1 and R^2 are independently selected from the group consisting of hydrogen, hydrocarbyl substituents of 20 carbon atoms or less, and oxyhydrocarbyl substituents of 20 carbon atoms or less containing 1 to 3 oxy groups, provided that R^1 and R^2 are not both hydrogen.

4. The oligonucleotide of claim 3 wherein one of R^1 and R^2 is hydrogen and the other is an oxyhydrocarbyl substituent having the structure $\text{CH}_3\text{O}-(\text{CH}_2)_x-$

wherein x is an integer in the range of 1 to 20, inclusive.

5 5. The oligonucleotide of claim 4 wherein x is 2 and the oxyhydrocarbyl substituent is 2-methoxyethyl.

6. The oligonucleotide of claim 3 wherein one of R^1 and R^2 is hydrogen and the other is a hydrocarbyl substituent is a straight-chain alkyl moiety having the
10 formula $CH_3(CH_2)_y-$ wherein y is an integer in the range of 0 to 15, inclusive.

7. The oligonucleotide of claim 6 wherein y is 11 and said hydrocarbyl substituent is dodecyl.
15

8. The oligonucleotide of claim 6 wherein y is 2 and said hydrocarbyl substituent is n-propyl.

9. The oligonucleotide of claim 1 wherein the
20 oligonucleotide is modified at both the 3' and the 5' termini.

10. The oligonucleotide of claim 2 wherein one of R^1 and R^2 is hydrogen and the other is a polymer.
25

11. The oligonucleotide of claim 2, wherein one of R^1 and R^2 is hydrogen and the other is a sugar moiety.

30 12. The oligonucleotide of claim 2, wherein one of R^1 and R^2 is hydrogen and the other is a chromophore.

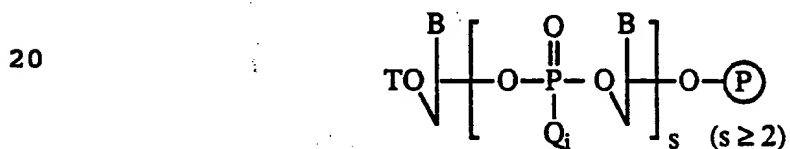
13. The oligonucleotide of claim 2, wherein one of R^1 and R^2 is hydrogen and the other is a steroid moiety.

5 14. The oligonucleotide of claim 1, wherein the number of said linkages is in the range of about 2 to 10.

10 15. The oligonucleotide of claim 1, further including 2 to 10 phosphorothioate linkages at either the 3' terminus, the 5' terminus, or both.

15 16. A method for synthesizing a polynucleotide stable to degradation by 3' exonucleases comprising the steps of:

(a) treating a nucleotide of the formula



25 wherein T is a protecting group, P is a protecting group or a solid state carrier, and each B is independently a protected or unprotected heterocyclic base, i varies from 1 to s; each Q_i is hydrogen or $-\text{NR}^1\text{R}^2$, provided that at least one Q_i is hydrogen; with an amine of the formula
 30 HNR^1R^2 wherein R^1 and R^2 are defined in Claim 1, in the presence of an oxidizing agent sufficient to form the product of the formula

35

- (b) removing the blocking group T,
- (c) condensing a 5'-blocked nucleoside
H-phosphonate, H-phosphorothioate or H-phosphoro-
5 dithioate, with the 5'-terminal hydroxy group of the
carrier bound nucleotide in the presence of an activating
agent;
- (d) removing the 5'-blocking group of the
5'-terminal nucleoside;
- 10 (e) sequentially repeating steps (c) and (d)
using 5'-blocked nucleoside H-phosphonate, H-phosphoro-
thioate and/or H-phosphorodithioate, until said
polynucleotide of desired length is obtained;
- (f) forming phosphodiester, phosphorothioate
15 and/or phosphorodithioate internucleotide linkages by
oxidation;
- (g) separating said polynucleotide from P.

20

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/03138

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL. (5): C07H 21/00; C07H 21/04

U.S. CL.: 536/27; 435/6

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴

Classification System

Classification Symbols

U.S. 536/27; 435/6

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁵

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category ⁶	Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	Nucleic Acids Research, Volume 14, No.8, issued 1986 (Oxford, England), Letsinger et al. "Effects of pendant groups at phosphorus on binding properties of d-ApA analogues," see pages 3487-3499, especially", pages 3493-3494.	1-16
A	Nucleic Acids Research, Volume 14, No. 13, issued 1986 (Oxford, England), Froehler et al., "Synthesis of DNA via deoxynucleoside H-phosphonate intermediates, see pages 5399-5407.	1-16
A	Nucleic Acids Research, Volume 16, No. 11, issued 1988 (Oxford, England), Froehler et al, "Phosphoramidate analogues of DNA; synthesis and thermal stability of heteroduplexes, see pages 4831-4839.	1-16

* Special categories of cited documents: ¹⁶

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ²

30 August 1990

Date of Mailing of this International Search Report ³

02 OCT 1990

International Searching Authority ¹

ISA/US

Signature of Authorized Officer ¹⁹

GARY L. KUNZ

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
A	Nucleic Acids Research, Volume 16, No.8, issued 1988, (Oxford, England), Stein et al., "Physicochemical properties of phosphorothioate oligodeoxynucleotides," see pages 3209-3221.	1-16
A	Tetrahedron Letters, Volume 27, No. 46, issued 1986, (Great Britain), Brian C. Froehler, "Deoxynucleoside H-phosphonate Diester Intermediates in the Synthesis of Internucleotide Phosphate Analogues," see pages 5575-5578.	1-16
A	Nucleosides and Nucleotides, Volume 6, No. 1 and 2, issued 1987, "The Use of Nucleoside H-phosphonates in the synthesis of Deoxyoligonucleotides", see pages 287-291 by Brian C. Froehler et al.	1-16